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We have previously established that IGF-I can stimulate chemotaxis of MCF-7 and MDA-231 human breast cancer cells. We now report that two closely related peptides, IGF-II and des(1-3)IGF-I are also capable of inducing directional migration in these two cell lines. Interestingly, neither is as potent as native IGF-I. Examination of two other growth factors, hepatocyte growth factor and TGF-α yielded markedly different results. While both were able to stimulate MDA-231 cells to migrate, TGF-α was found to be the first non-IGF mitogen able to induce a significant response in the MCF-7 cells. Migration assays done with anti-integrin antibodies revealed that the α3 subunit is not required for MCF-7 cells to migrate through Type IV collagen. However, α3 plays some role in the MDA-231` cells' migration through laminin. Exposure to calf serum does not increase the number of Type I IGF receptors in the MCF-7 cells, as assessed by IGF-I-crosslinking studies. Differences in the amounts of IGFBPs secreted by MCF-7 cells may partially explain the enhanced migration seen after calf serum exposure. Pre-incubation with IGFBP-1 did not inhibit MCF-7 migration to IGF-I. The effect of IGFBP-1 on MDA-231 cell migration will require further study for a definitive conclusion to be drawn.

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Monica E. Dorr, MD
PI - Signature

7/27/94
Date

The role of Integrins and IGFBPs in the IGF-I Stimulated Migration of Human Breast Cancer Cells

Task 1 Months 1-6: Complete migration assays with MDA-231 and MCF-7 cells with laminin, collagen (types I and IV) and fibronectin using IGF-I, EGF, FGF, PDGF and hepatocyte growth factor. Compare the response to des 1-3 IGF-I and IGF-II to IGF-I. Continue work to complete the analysis of the response to EGF, FGF and PDGF using each of the 4 matrices. This should be completed by May 1998.

Task 2 Months 5-16: Perform immunoprecipitation of the integrin studies on four additional candidate cell lines, then conduct assays to determine:

a. whether IGF-I stimulates their migration and compared IGF-I's effects to those of other growth factors.
b. whether the increased migration, if present, is due to chemotaxis
c. study the effects of anti-integrin antibodies, using the pattern of integrin expression for each cell as a guide

This work is progressing well at present. The integrins that are present in the four additional cell lines have been defined. This should be completed by September, 1998.

Task 3 Months 6-24: Study the calf serum effect on MCF-7 cells in greater detail by performing:

a. cross-linking studies for IGF-I receptors.
b. migration assays with cells grown in calf serum with supplemental IGF-I.
c. migration assays with cell growth in media containing tamoxifen and other anti-estrogens.
d. ligand blots and immunoblots on conditioned media from cells with various calf serum exposure times to determine any changes in IGFBP expression.

Task 4 Months 16-36: Determine the effects of IGFBP-1 through BP-5 on IGF-I stimulated migration:

a. add each IGFBP to the upper and lower chambers immediately prior to migration assays.
b. perform migration assays with several different breast cancer cell lines grown in media containing a supplemental amount of each IGFBP
c. use IGF-I analogs with decreased IGFBP affinities to study IGF-I independent effects

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INTRODUCTION

Insulin-like growth factor-I (IGF-I, formerly known as somatomedin C) is one of the broad spectrum polypeptide growth factors with biological actions in diverse tissues (1). The amino acid sequence of IGF-I has 48% homology with human proinsulin (2). When IGF-I binds to its receptor, the 185 kilodalton (kDa) protein known as insulin substrate-1 (IRS-1) undergoes tyrosine phosphorylation. IRS-1 mediates cellular responses to stimulation of both IGF-I and insulin receptors (3). A study published in 1984 first established that IGF-I stimulated the growth of human breast cancer (HBC) cells kept in long-term tissue culture (4) and physiologic levels of IGF-I stimulate DNA synthesis in the absence of other serum components. This study also showed that the HBC cell membranes have IGF-I receptors (4). Later studies performed on breast cancer biopsy specimens evaluated IGF-I receptors and found that the cancerous tissue had higher levels of this receptor than did adjacent normal tissue from the same specimen (5).

Stewart et al. (6) discovered that estradiol sensitizes MCF-7 cells to the mitogenic effects of IGF-I by increasing mRNA for the IGF-I receptor. Subsequent studies (7-9) have examined the effects of αIR-3, a murine monoclonal antibody to the IGF-I receptor. This antibody significantly limited growth of both MCF-7 and MDA-231 cells *in vitro* (8).

Several authors have reported that IGF-I can stimulate migration of malignant cells, including human melanoma (10) cells and pancreatic carcinoma (11) cells. These experiments and others utilized a modified microwell Boyden chamber. Because it is necessary in the early stages of the metastatic process for tumor cells to bind to and migrate through basement membranes to gain access to the circulatory system, use of the Boyden chamber allows for study of the invasive capacity of cancer cells.

Others have studied malignant breast cells in Boyden chamber assays. Verhasselt and colleagues (12) found that interleukin-1 and interleukin-6 stimulate migration of SK-BR-3 and ZR-75-1 cells. Hansen et al. (13) studied the effects of 1α,25 dihydroxyvitamin D3 on MDA-231 cells. They found that after a seven day exposure to $1\alpha 25(OH)_2D_3$, the invasive capacity of these cells was markedly reduced. Interpretation of Hansen's results is complicated by the fact that her group used Matrigel™, which contains IGF-I at a 15 ng/ml concentration to coat their membranes.

Given the proven mitogenic effects of IGF-I on HBC cells and its ability to induce migration in other malignant cell types, our work began with the hypothesis that IGF-I would stimulate migration of breast carcinoma cells. We selected MCF-7 and MDA-231 as representative of ER positive and ER negative breast cancer cells, respectively. Our initial studies were published in February of 1996 in the Journal of Biological Chemistry (JBC) (14). We found that IGF-I stimulated migration in both cell lines. The conditions required for the MCF-7 cells to migrate in response to IGF-I were much more specific than those for the MDA-231 cells. The MCF-7 cells would only migrate through type IV collagen or vitronectin-coated membranes. (Fig. 1, ref. 14). Interestingly, they do not migrate in response to their growth media, which contains 10% fetal calf serum.

Once a migratory response to IGF-I had been established, we conducted checkerboard assays to assess the relative amount of chemotaxis (directional migration) versus that of chemokinesis (random movement) caused by IGF-I. These studies (Fig. 4, ref. 14) revealed that the majority of increased cellular migration was, in fact, directional towards IGF-I. Marked inhibition of IGF-I-stimulated migration was seen in both cell lines when they were pre-

incubated with an anti-IGF-I receptor antibody (termed αIR3), confirming the necessity of normal receptor function. Additional studies were done to compare IGF-I's effect with that of other known and likely chemoattractants. The MDA-231 cells demonstrated a similar degree of stimulation by epidermal growth factor (EDF), but the effects of platelet deprived growth factor (PDGF-BB) and fibroblast growth factor (FGF) were not as pronounced (Fig. 3, ref. 14). In contrast, the MCF-7 cells had essentially no response to any other chemoattractant, regardless of the membrane coating substance used.

Within the past few years, the role of integrins in cellular motility and invasion has been recognized. The integrins are a superfamily of heterodimeric cell-surface glycoprotein receptors composed of distinct α and β subunits. Adhesion of cells to their extracellular matrix (ECM) is primarily mediated by these receptors; furthermore, integrins have a central role in transducing signals from the extracellular environment. Immunohistochemical studies have demonstrated that breast carcinoma tissue has altered integrin expression compared to normal breast epithelium (15). Coopman et al. (16) examined the effects of various basement membrane (BM) components on directional migration of MCF cells on glass coverslips. They found that laminin arrested migration of one MCF-7variant while collagen type IV and fibronectin had no effect. A follow-up study by the same group (17) found no involvement of a 67 kD high-affinity laminin receptor (which is not an integrin) in this phenomenon. Since the MCF-7 cells express a variety of integrins capable of binding laminin, this study raised the possibility that these receptors mediate this effect. Using a hydrated collagen gel containing heterotypic cell populations including MCF-7 cells, Rossi et al. (18) found that the presence of fibroblasts was necessary for MCF-7 cells to migrate. It has been shown that fibroblasts secrete IGF-I (19) therefore; this requirement of MCF-7 cells may actually reflect the cells' need for IGF-I in the setting of migration. Using a different cell line, 8701-BC (isolated from a highly invasive ductal infiltrating carcinoma), Luparello and colleagues (20) found that type I-trimer collagen had a permissive effect on cell locomotion.

Two papers published in 1994 established links between vitronectin receptors and growth factor signaling. Vuori and Rusolahti (21) reported that stimulation of rat fibroblasts with insulin led to association of the $\alpha V\beta 3$ integrin (a vitronectin receptor) with IRS-I. As discussed earlier, IRS-1 is an intracellular protein which mediates signaling by both insulin and IGF-I. This publication also utilized FG human pancreatic carcinoma cells which do not normally express the alpha V beta 3 ($\alpha V\beta 3$) integrin and found that transfecting the FG cells with $\beta 3$ integrin complementary DNA increased their response to insulin. Specifically, the authors stated that the transfected cells expressing $\alpha V\beta 3$ integrin responded to insulin with a 2.5-fold higher level of DNA synthesis when grown on vitronectin compared to collagen. Cells without the $\alpha V\beta 3$ integrin had the same response to insulin regardless of the plating substance used. The doses of insulin used in these studies (up to100 nM) are sufficient to cause stimulation of IGF-I receptors. The relative contribution of insulin receptors versus
IGF-I receptors was not investigated.

A second study by Klemke et. al (11) also using the FG cells found that the epidermal growth factor (EGF), was necessary for these cells to migrate on vitronectin. The FG cells utilize the $\alpha V\beta 5$ integrin for attachment to vitronectin, and this process does not require EGF. However, tyrosine kinase signaling by the EGF receptor was required for their migration on vitronectin but not on collagen. These cells display constitutive migration on collagen which is not affected by the EGF receptor specific tyrosine kinase inhibitor tyrphostin 25. This same substance prevents EGF-induced FG cell migration on vitronectin. As discussed below, this association between a growth factor and integrin signaling as a prerequisite for migration is

almost certainly present with MCF-7 cells as well. Our laboratory performed immunoprecipitation studies to characterize the pattern of integrin expression in the MDA-231 and MCF-7 cells (Fig. 2, ref 14). Numerous migration assays done with anti-integrin antibodies have confirmed that integrins play a key role in IGF-I stimulated migration. Furthermore, the effects of anti-integrin antibodies are highly specific for the coating substance used and vary with the relative amounts of integrins expressed by a given cell line (Fig. 6, ref. 14).

Included in our original grant proposal was preliminary data related to the effect of calf serum exposure on MCF-7 cells. We found that MCF-7 cells maintained in 10% fetal calf serum (FCS), lost their capacity to migrate in response to IGF-I. Remarkably, the same MCF-7 cells, which had shown no migration, could be grown under identical conditions and split into two different plates--one containing 10% fetal calf serum and the other containing 10% calf serum. Ten days later, typically after two more passages, the cells in the calf serum would migrate well when stimulated with IGF-I while the group of cells continued in fetal calf serum would not migrate in any measurable numbers. No such loss of migratory ability was observed in the MDA-231 cells maintained for several months in 10% FCS. To determine the time period of exposure to calf serum that was necessary for cellular migration, an assay was done with seven separate populations of MCF-7 cells grown under different circumstances--six populations had been exposed to calf serum for varying periods of time and a control population which had always been in fetal calf serum. This assay revealed that exposure to calf serum for seven to ten days was associated with the most active migration. Interestingly, cells which had been in calf serum for over a month demonstrated nearly the same poor migratory response to IGF-I as cells which had always been maintained in fetal calf serum. As part of the work proposed in our grant, we performed cross-linking studies on MCF-7 cells grown in both 10% FCS and 10% calf serum.

The biologic activities of IGF-I are largely regulated by a group of high affinity IGF binding proteins (IGFBPs), six of which have been cloned and sequenced. These IGFBPs have negligible affinity for insulin and are present in the circulation and throughout the extracellular space. They are also found in the conditioned media and extracellular matrix of most cells in culture. These binding proteins have at least four major functions, which include acting as transport proteins and prolonging the half-life of IGF-I. In addition, they provide a means for IGF localization to specific tissues and cell types and directly modulate the interactions of IGF with its receptor (I). The role of these IGFBPs in HBC cell growth and metabolism has been an area of intense investigation in the past several years.

One published study reported a correlation between ER status and IGFBP secretion in breast cancer cell lines. It showed that ER negative cells secrete IGFBP-1 and IGFBP-3. In contrast, the ER positive lines were found to secrete IGFBP-2 and IGFBP-4 (22). Subsequent studies sought to understand how IGFBPs affect the growth of HBC cells. One study demonstrated that IGF-I increased cell numbers of sparse MDA-231 cultures by 24% after 48 hours (23). Addition of IGFBP-1 led to a 45% increase while IGFBP-2 had no potentiating effect. However, IGFBP-1 in the absence of IGF-I had no effect on cell growth. A different group examined the actions and IGF-I and IGFBP-1 on MCF-7 cells. The MCF-7 cells normally require estrogen for growth; nevertheless, IGF-I can substitute for estrogen in short-term studies. In contrast to the findings for MDA-231 cells, IGFBP-1 was shown to block IGF-I induced mitogenesis in MCF-7 cells (24). After the identification of a sixth IGFBP, Sheikh et al. (25) examined four ER positive and three ER negative HBC cell lines for expression of IGFBP-5 and IGFBP-6. All cell lines except the MDA-231 expressed IGFBP-5 mRNA. In 1994 Chen et al. (26) reported that effects of multiple IGFBPs on IGF-stimulated DNA synthesis in MCF-7 cells.

They found that IGFBP-2 and IGFBP-3 enhanced DNA synthesis in response to IGF-I while IGFBP-4 and IGFBP-5 had no effect.

The expression of IGFBPs has also been examined in breast cancer tissues, where the correlation with ER status is not as clear-cut. Pekonen et al. (27) found that only IGFBP-3 expression could be correlated to ER status, being more common in ER negative tumors. They discovered that each of the tissue specimens, regardless of ER status, expressed all five of the IGFBPs that were known to exist at the time of publication. A more recent 1994 study by McGuire et al. (28) performed ligand blots for IGFBPs on eighty breast cancer tissue specimens. They were unable to detect IGFBP-1 in any of the specimens. A positive correlation of ER expression and IGFBP-4 expression was found, while IGFBP-3 was negatively correlated with ER status.

Two of the IGFBPs have been shown to have direct effects independent of IGF-I. In 1989, Blat et al. (29) reported that IGFBP-3 (then known as inhibitory diffusible factor 45) can inhibit serum stimulation of DNA synthesis in the chick embryo fibroblast cell line. Later, Jones et al. (30) found that IGFBP-1 stimulates migration of the Chinese hamster ovary (CHO) cells in woundings assays even in the absence of IGF-I. A single amino acid substitution in the IGFBP-1 sequence which prevented its binding to the $\alpha 5\beta 1$ integrin on the CHO cell surface reversed this stimulation. Oh and co workers (31) reported that IGFBP-3 has IGF-receptor independent activities in an ER negative cell line, Hs578T. These authors found that treatment of the Hs578T cells with exogenous IGFBP-3 significantly inhibited their monolayer growth. This inhibitory effect could be overcome by native IGF-I, but not by IGF analogs with normal affinity for the IGF-I receptor but decreased affinity for IGFBP-3. This finding suggested that the effect was independent of the IGF-I receptor but was blocked by the IGF-IGFBP-3 interaction (20).

BODY

EXPERIMENTAL METHODS

Migration Assays - The Boyden chamber migration assays were performed by filling the lower wells with a putative chemoattractant or control solution, over which the coated polycarbonate membrane (pore size-8 μm) is placed. Phenol-red free DMEM/F12 with 0.01% bovine serum albumin (BSA) is used as a negative control solution, and all growth factors are prepared in this solution. Monolayers of confluent or near confluent cells are trypsinized and re-plated twelve to sixteen hours prior to each assay. Each assay begins by rinsing the cells with PBS/EDTA (phosphate buffered saline containing a 5 mM concentration of EDTA) and separating them from the tissue culture dish with 2 ml of 0.1X trypsin in PBS/EDTA. Then 8 ml of a 10% serum containing media are added to inactivate the trypsin. The cell suspension is centrifuged once for 10 minutes; the serum containing media is then removed. Next the cells are resuspended and washed with the DMEM/BSA solution twice. After the second centrifugation, the supernatant is removed. The cell pellet is then re-suspended in a final volume of one to three ml. A hemacytometer is used to estimate the density of the cell suspension.

For those experiments in which an anti-integrin antibody is used, the cells are separated into microcentrifuge tubes and incubated with the antibody by gently tumbling at room temperature for thirty to sixty minutes. All anti-integrin antibodies were used at a concentration of 25 μg/ml. For those experiments in which IGFBP-1 or its [221Tryp] mutant were used, the BP-1 (final concentration 10 nM or 50 nM) was added to the cell suspension. The suspension was then tumbled at room temperature for 45-60 minutes. A 50 μl volume of the suspension containing approximately 10,000-15,000 cells (MDA-231) or 50,000-60,000 cells (MCF-7) is loaded into each upper well, and the chamber is placed in a 37°C 5%CO2-enriched incubator. After a four hour period, the chamber is disassembled. The cells adherent to the upper surface of the membrane are scraped away so that only those cells which have migrated through the membrane remain. The membrane is then fixed in methanol, stained with Diff-Quik and allowed to air dry on a glass slide. A grid eyepiece in our microscope is then used to reliably demarcate several areas within each individual well in order to count migrated cells. Two full grids are counted for each of the MDA-231 cell wells and four grids are counted for each MCF-7 well.

Cross-linking Studies - Two populations of MCF-7 cells were studied, those which have been exposed to calf serum(CS) for seven to ten days and those which have always been in fetal calf serum (FCS). Both groups were trypsinized and replated in the appropriate medium in a six-well plate twelve to sixteen hours prior to cross-linking. This sequence of events reproduced the conditions of under which MCF-7 cells migrate well to IGF-I. The cells were rinsed twice with binding buffer (HCO3-free EMEM with 0.1% BSA and 20 mM Hepes at pH 7.3), then incubated with 3 to 10 μCi of ^{125}I-IGF-I with increasing concentrations of unlabelled IGF-I. Following a 90 min incubation at 4 °C, the wells were be aspirated and rinsed twice with cross-linking buffer, which contained EMEM without amino acid with 20mM Hepes at pH 7.3. Immediately prior to cross-linking, freshly prepared 10 mM DSS in DMSO was added to the cross-linking buffer to

yield a final concentration of 0.5 mM. The cells were then incubated in the DSS in cross-linking buffer for 30 min at 4°C. The DSS was then aspirated and neutralized with 0.1 M Tris-HCl with 0.1M NaCl at pH 7.4. The wells were rinsed twice with this neutralizing buffer, and received a third rinse of 5 to 10 min duration. The buffer was aspirated and the cells were lysed in minimal volumes SDS sample buffer with 100mM DTT (dithiothreitol) to achieve reducing conditions. Standardized samples were resolved in a 6% SDS-PAGE.

RESULTS

Task 1 - In order to more comprehensively understand that possible role of other growth factors in altering the IGF-I mediated chemotaxis of breast cancer cells, several other factors were studied. These included IGF-II, des-1 through -3 IGF-I (a form of IGF-I that does not bind to IGF binding proteins), TFG-α, and transforming growth factor beta (TFGβ) as well as hepatocytes growth factor. IGF-II is particularly important since it is produced by human stromal breast tissue. The results of four independent experiments with des-IGF-I as compared to native IGF-I were conducted in both MDA-231 cells and MCF-7 cells as shown in Table 1 on page 14 of this report. Des-IGF-I was consistently less potent than IGF-I at all concentrations tested. However, there was stimulation over background with all concentrations of des-IGF-I. When the degree of stimulation in several experiments is averaged, the response to 10 ng.ml of des-IGF-I was consistently 70% as potent as native IGF-I. The suggests that IGF-I binding to IGFBPs is enhancing the effect of native IGF-I as compared to the des-IGF-I since the affinities for the IGF-I receptor are identical.

The results from three experiments comparing the stimulatory effect of IGF-II are summarized in Table 2. The response of both of these breast tumor cell types to IGF-II was more variable; however, IGF-II consistently stimulated chemotaxis over baseline. In most, not all of the assays the response to IGF-II was less than IGF-I. This is consistent with the fact that IGF-II has a lower affinity for the IGF-I receptor. As shown in Table 3, hepatocyte growth factor or scatter factor is also a stimulation of migration. When the response to 10 ng/mlg of this factor was compared to IGF-I the responses were between 21 and 61% of those induced by 10 ng/ml of IGF-I. In all but one experiment, hepatocyte growth factors less potent than IGF-I. Transforming growth factor-α (TFG-α) was equipotent with IGF-I at relatively low concentrations of 1 ng/ml but unlike IGF-I, no increase was noted when concentrations between1 and100 ng/ml were utilized, as shown in Table 4. In MDA-231 cells and MCF-7 cells, concentrations of 10 and 100 ng/ml were less potent than IGF-I, but did cause some stimulation of chemotaxis. TGF-β was considerably less potent. The response of cells was weak and only a small fraction of the cells were stimulated (e.g. $<$20% of the response to 10 ng/ml IGF-I) in spite of the fact that concentrations as high as 250 ng/ml of TGF-β were tested. Studies to determine the effect of EGF FGF and PDGF are currently being completed.

A second major component of the work in Task 1 was to determine if other integrins were important in mediating the breast tumor cell migration response to IGF-I. In nearly all of these experiments, Type IV collagen was used as substratum. However, to test the effects of anti-α3 integrin antibodies, laminin was used as a substratum component in the some experiments. The effect of anti-α2 and anti-β1 antibodies on migration through Type IV collagen are summarized in Figure 6B of reference 14. The results appear to show that the $\alpha 2\beta 1$ integrin is the key integrin for migration of these cells through Type IV collagen. The data also show that an anti-α2 antibody inhibited the number of cells migrating in response to IGF-I across a Type IV collagen matrix by approximately 48%. In contrast, as shown in Table 5 of this report,

anti-α3 antibody was stimulatory for the MCF-7 cells, but an anti-β1 antibody was very inhibitory. Unlike MCF-7 cells, when MDA-231 cells were plated on laminin, it did not inhibit their migration. Anti-α2 and anti-α3 antibodies were weakly inhibitory causing approximately 15% inhibition over the basal migration response to IGF-I. In contrast, the β1 antibody completely blocked migration suggesting again that β1 is the key integrin for this cell type. The MDA-231 cells have a relatively higher number of $\alpha3\beta1$ receptors compared to MCF-7 cells. This could be the major integrin mediating IGF-stimulated migration through laminin. In one experiment with the MDA-231 cells, the anti-α3 antibody produced 42% inhibition which strengthens this conclusion.

In summary, the data relative to Task 1 shows that we have made substantial progress toward completion of this task in defining the major integrins and growth factors that mediate migration of these cells in the culture and that work cooperatively with IGF-I to enhance its effects on migration.

Task 2- This could not be undertaken because Dr. Doerr became pregnant in October of 1996. This made it impossible for her to utilize the concentrations of radioactive substances that would have been required to complete this work. The UNC Radiation Safety Office specifically forbid her to work with this level of radioactively. Therefore, these studies will have to be completed in future years when the radiation risk is not present. We initiated these studies in 10/97. However work has been completed that define the integrins that are present on each of the four additional cell lines. They are as follows:

Cell Line	T47D	BT20	HS578T	ZR-75-1
	$\alpha2\beta1$	$\alpha V\beta1$	$\alpha2\beta1$	$\alpha2\beta3$
	$\alpha V\beta3$	$\alpha32\beta1$	$\alpha6\beta1$	$\alpha V\beta5$
	$\alpha V\beta1$	$\alpha5\beta1$	$\alpha V\beta1$	$\alpha5\beta1$
	$\alpha5\beta1$	$\alpha V\beta3$	$\alpha3\beta1$	$\alpha3\beta1$
	$\alpha V\beta5$			$\alpha2\beta1$

This information should help to reflect the correct substrata for future experiments.

Task 3 - We completed the affinity crosslinking studies that were proposed in this section. A representative autoradiograph of a gel from this work is shown in Figure 1 on page 19. Lanes 1-4 contain MCF-7 cells grown always in 10% fetal calf serum. Lanes 5-8 contain cells exposed to 10% calf serum for 10 days. The band corresponding to approximately 133 kDa represents the Type I IGF receptor crosslinked to radiolabelled IGF-I. The cells loaded in lanes 2 and 6 were exposed to a high concentration of insulin
(5 μg/ml) as a control. They show no obvious difference in the number of Type I receptors between fetal calf serum and calf serum exposed MCF-7 cells. That is, analysis of the Type I IGF receptor abundance as assessed by affinity crosslinking shows that the receptor band is of equal intensity whether the MCF-7 cells are cultured in fetal calf serum or calf serum. This is important because in order to retain IGF-I responsiveness, these cells need to be cultured in calf serum and this eliminates the possibility that this difference if due to a significant change in receptor number.

We also analyzed the medium produced by cells that have been culture in either calf serum or fetal calf serum for IGFBP content. Phosphor Image analysis of a Western ligand blot

showed that cells cultured in calf serum were secreting lower levels of IGFBPs compared to cells cultured in fetal calf serum. This allows the cells to be more responsive to IGF-I since, in general, IGFBPs that are produced by breast cancer cells appear to be inhibiting their migratory response. Therefore, it is probable that IGFBPs are inhibitory and the lower levels of the IGFBPs present in the conditioned medium obtained from cells cultured in calf serum that secrete lower levels of IGBFPs would allow an enhanced migratory response.

Task 4- To date, we have tested the effects of one IGF binding protein (e.g. pure IGFBP-1) on migration of MCF-7 cells. We also tested a mutant form of this protein that has had its RGD sequence mutated to WGD. This results in decreased ability of this substance to directly stimulate that migration of CHO cells and smooth muscle cells (SMC) (30). The concentration of IGFBP-1 used in the earlier assays was 10 nM, as this concentration has been reported to inhibit the IGF-I stimulated growth of MCF-7 cells (24). Later assays used a concentration of 50 nM IGFBP-1 since unpublished observations by investigators in our lab have found that a molar excess of IGFBP-1 relative to IGF-I is necessary for producing a significant effect. As seen in Table 6 on page 20 of this report, neither concentration of the wild-type protein stimulated migration of MCF-7 cells in the absence of IGF-I. Furthermore, IGFBP-1 had no inhibitory effect on the cellular response to IGF-I. The mild stimulatory effect of IGFBP-1 in combination with IGF-I is probably not statistically significant.

When a 10 nM concentration of IGFBP-1 was tested in the MDA-231 cells (Table 7), the binding protein initially appeared to have some intrinsic stimulatory activity and enhanced that response to IGF-I. This was seen in two separate experiments. The final assay listed on Table 7, which used a 50 nM concentration of IGFBP-1 produced conflicting results. This assay utilized a separate aliquot of IGFBP-1, but this aliquot was from the same collection as was used for the earlier experiments. Results obtained with the WGD mutant pre-incubated with both the MCF-7 and MDA-231 cells are summarized in Table 8 on page 22. These studies suggest that IGF binding proteins may have modulatory effects on cell responsiveness and mandate that we conduct further studies in the future to investigate these possibilities.

DISCUSSION

The major finding of these studies is that other growth factors other than IGF-I can stimulate chemotaxis of these cells. However, of the other growth factors tested, none was more potent than IGF-I. Of note, TGF-α was the only non-IGF-family chemoattractant able to induce migration in the MCF-7 cells. Distinct combinations of growth factors were not tested although combinations such as hepatocyte growth factor and IGF-I may be synergistic in terms of stimulating chemostaxis. However, when tested in isolation, the cells consistently respond to IGF-I better than these other growth factors except for TFG-α which was equipotent. It elicited the same maximal response and it was more potent on a molar basis.

A secondary finding of these studies was that des-IGF-I was significantly less potent than IGF-I. This is consistent with previously published findings which have shown that certain forms of IGF binding proteins can augment the response of breast tumor cells in culture to IGF-I. These include IGFBP-1 for MDA-231 cells and IGFBP-3 for MCF-7 cells. However, IGF binding proteins have also been shown to inhibit cell migration in smooth muscle cells and to inhibit DNA synthesis in breast carcinoma cells. Therefore, determining the exact conditions under which IGF binding proteins might facilitate cell response to IGF-I versus inhibition needs further experimental analysis. In the experiments conducted in task 4, we noted some modest

stimulation of migration of MDA-231 cells by IGFBP-1 and some accentuation of the response to IGF-I. Therefore, it would be not surprising with these cells if des-IGF-I might be less potent than IGF-I. However, the exact explanation for the lack of equipotency in MCF-7 cells has not been definitively determined. Clearly, since breast carcinoma cells *in vivo* produce IGF binding proteins, experiments directed to determine how they might augment the cell migration response to IGF-I would be important for future studies.

Our studies further extend our published observations regarding integrin cooperativity with the IGFs. Specifically, these studies show the $\alpha 2\beta 1$ integrin is probably the key integrin for migration of these cells over a Type IV collagen matrix. This matrix is relevant to breast carcinoma tissue *in vivo* since this is one of the major substituents of extracellular matrix for this cell type. Both $\alpha 2$ and $\beta 1$ antibodies inhibit the migration of MDA-231 cells through a Type IV collagen basement membrane. However, anti-$\alpha 3$ antibodies were inhibitory since the $\alpha 3\beta 1$ integrin is also present on MDA-231 cells. In contrast, the MCF-7 cells do not have $\alpha 3$ integrin and therefore, probably migrate across this matrix component using $\alpha 2\beta 1$. These observations clearly lead to suggestive future experiments. In other cell types, we have noted cooperativity between the $\alpha V\beta 3$ integrin and IGF-I receptor signaling system. In those studies, we have been able to show that phosphorylation of key signal transduction proteins such as IRS-1 are blocked when ligand occupancy of $\alpha V\beta 3$ is blocked. These findings suggest this type of experiment should be conducted with $\alpha 2\beta 1$ and $\alpha 3\beta 1$. Since we have specific antibodies that can block ligand occupancy of these receptors, future studies should be directed toward determining whether such blockage inhibits IGF-I receptor-induced phosphorylation of IRS-1 or IRS-2, the principal signal transduction components for this receptor.

Our studies did not definitively show why our MCF-7 cells require culture in calf serum as opposed to fetal calf serum to maintain IGF-I responsiveness. The lower level of IGFBPs in calf serum culture medium is suggestive, but not definitive, particularly in light of the observations that we made with des-IGF-I and direct addiction of IGFBP-1. However, it s still possible that inhibitory binding protein such as IGFBP-4 is more abundant in the fetal calf serum exposed cells and this is negating the other effects noted herein. More exhaustive work characterizing the actual rates of biosynthesis of various forms of IGF binding proteins in the calf serum versus fetal calf serum exposed cells might shed some light on this subject.

The role of IGFPBPs also needs further definitive evaluation. IGFBP-1 is but one of six members of this family and therefore, we have not evaluated by direct addiction experiments the roles of IGFBP-2 through -6. Since various breast epithelial cells have been shown to synthesize several of these proteins, principally IGFBP-2, -4, -5. We need to determine the relative roles in changing the chemotaxic response to IGF-I. Likewise, since two of these proteins (IGFBP-3 and IGFBP-5) are sequestered in ECM, we also need to determine the roles of matrix-sequestered IGFBPs in modulating these effects.

In summary, we believe the first year of these studies has made good progress toward completion of several of the tasks. However, more definitive studies to biochemically characterize the changes that are occurring in the IGF-I/IGF-I receptor and IGF binding proteins as well as signal transduction elements need to be undertaken and will be undertaken during the second year of these studies.

CONCLUSIONS

1. Within the IGF family, IGF-I is the most potent chemoattractant for stimulation of migration for MCF-7 and MDA-231 cells. The MDA-231 cells respond to a number of other

chemoattractants, such at EGF, HGF and TGF-α. In contrast, the MCF-7 cells respond only to members of the IGF family and TGF-α.

2. Integrins play an important roles in IGF-I stimulated migration. For migration through Type IV collagen and laminin, normal function of the β1 integrin is essential. Among the α subunits, α2 is important for migration through Type IV collagen whereas α3 appears not to be involved. For the MDA-231 cells, α3 is probably playing a part in their successful migration through laminin.

3. The explanation for the improved migration of MCF-7 cells after exposure to calf serum is not due a marked increase in Type I IGF receptors; crosslinking studies failed to show any appreciable difference in the number of receptors. Differences in IGFBP levels may be involved; this area requires further investigation.

4. In contrast to the inhibitory effect of IGFBP-1 on MCF-7 cells' IGF-stimulated mitogenesis shown by others, IGFBP-1 showed no ability to inhibit MCF-7 cells' migratory response to IGF-I. The effects of IGFBP-1 on MDA-231 cells and the role of direct effects of IGFBP-1 on breast cancer cell membranes as assessed by comparison with the WGD mutant form of IGFBP-1 can not yet be stated with certainty. Further study using additional IGFBPs is clearly needed.

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TABLE 1

Migration Response of Human Breast Cancer Cells to Des(1-3)IGF-I

	MDA-231 cells	MCF-7 cells
Assay #106		
Response to:		
native IGF-I at 10 ng/ml	415 (100%)	657 (100%)
des-IGF-I at 10 ng/ml	354 (85%)	304 (46%)
Assay #107 all MCF-7 cells	des- IGF-I	native IGF-I
Response to:		
10 ng/ml	266 (84%)	317 (100%)
30 ng/ml	123 (39%)	246 (78%)
100 ng/ml	86 (27%)	85 (27%)
Assay #108 all MCF-7 cells	des- IGF-I	native IGF-I
Response to:		
3 ng/ml	255 (68%)	319 (85%)
10 ng/ml	185 (49%)	377 (100%)
30 ng/ml	188 (50%)	362 (96%)
Assay #113 all MCF-7 cells	des- IGF-I	native IGF-I
Response to:		
10 ng/ml	331 (72%)	457 (100%)
30 ng/ml	244 (53%)	not done
100 ng/ml	139 (30%)	not done
300 ng/ml	170 (37%)	not done

Table 1. Results from assays using des(1-3)IGF-I. The migration to IGF-I at 10 ng/ml is arbitrarily assigned a value of 100% . All data represents actual number of cells counted followed by (%), percentage migration compared to that observed in response to native IGF-I at 10 ng/ml.

TABLE 2

Migration Response of Human Breast Cancer Cells to IGF-II

	MDA-231 cells	MCF-7 cells
Assay #98		
Response to:		
native IGF-I at 10 ng/ml	302 (100%)	473 (100%)
IGF-II at 10 ng/ml	not done	281 (59%)
IGF-II at 30 ng/ml	308 (100%)	398 (84%)

	MDA-231 cells	MCF-7 cells
Assay #99		
Response to:		
native IGF-I at 10 ng/ml	207 (100%)	221 (100%)
IGF-II at 10 ng/ml	not done	212 (96%)
IGF-II at 30 ng/ml	132 (64%)	170 (77%)

Assay #102 all MCF-7 cells	IGF-II	native IGF-I
Response to:		
10 ng/ml	219 (61%)	358 (100%)
30 ng/ml	474 (132%)	not done
100 ng/ml	261 (73%)	not done
300 ng/ml	57 (16%)	not done

Table 2. Results from assays using IGF-II. The migration to IGF-I at 10 ng/ml (the concentration used for all assays reported in this table) is arbitrarily assigned a value of 100%. All data represents actual number of cells counted followed by (%), percentage migration compared to that observed in response to native IGF-I at 10 ng/ml.

TABLE 3

Migration Response of Human Breast Cancer Cells to HGF/SF

Assay #135 all MCF-7 cells Response to:	HGF/SF	IGF-I
10 ng/ml	92 (20%)	467 (100%)
50 ng/ml	97 (21%)	not done

Assay #140 all MDA-231 cells Response to:	HGF/SF	IGF-I
5 ng/ml	474 (132%)	not done
10 ng/ml	219 (61%)	358 (100%)
50 ng/ml	261 (73%)	not done
100 ng/ml	57 (16%)	not done

Assay #143 all MDA-231 cells Response to:	HGF/SF	IGF-I
5 ng/ml	608 (68%)	not done
10 ng/ml	529 (59%)	892 (100%)
50 ng/ml	678 (76%)	not done
100 ng/ml	761 (85%)	not done

Table 3. Results from assays using Hepatocyte Growth Factor/Scatter Factor (HGF/SF). The migration to IGF-I at 10 ng/ml (the concentration used for all assays reported in this table) is arbitrarily assigned a value of 100% for each assay. All data represents actual number of cells counted followed by (%), percentage migration compared to that observed in response to native IGF-I at 10 ng/ml. Note: MCF-7 cells were used in two additional assays, #137 and #139, but they showed no response to HGF/SF in these experiments.

TABLE 4

Migration Response of Human Breast Cancer Cells to TGF-α

Assay #144 all MCF-7 cells	TGF-α	IGF-I
Response to:		
10 ng/ml	274 (63%)	433 (100%)
100 ng/ml	253 (58%)	not done

Assay #147 all MCF-7 cells	TGF-α	IGF-I
Response to:		
5 ng/ml	174 (36%)	not done
10 ng/ml	320 (66%)	487 (100%)
50 ng/ml	399 (82%)	not done
100 ng/ml	326 (67%)	not done

Assay #148 all MCF-7 cells	TGF-α	IGF-I
Response to:		
10 ng/ml	301 (67%)	451(100%)
100 ng/ml	313 (69%)	not done

Assay #147 all MDA-231 cells	TGF-α	IGF-I
Response to:		
1 ng/ml	871 (101%)	not done
5 ng/ml	807 (94%)	not done
10 ng/ml	918 (107%)	861 (100%)
100 ng/ml	798 (93%)	not done

Table 4. Results from assays using Transforming Growth Factor-α (TGF-α). The migration to IGF-I at 10 ng/ml (the concentration used for all assays reported in this table) is arbitrarily assigned a value of 100% for each assay. All data represents actual number of cells counted followed by (%), percentage migration compared to that observed in response to native IGF-I at 10 ng/ml.

TABLE 5
Effects of anti-α3 Antibody on IGF-I Stimulated Migration

Assay #110 all MCF-7 cells, migrating to IGF-I at 10 ng/ml through Type IV Collagen

Antibody used	Cells migrating
none	232 +/- 41 (100%)
anti-α2	112 +/- 11 (48%)
anti-α3	302 +/- 39 (130%)
anti-β1	74 +/- 5 (32%)

Assay #112 all MCF-7 cells, migrating to IGF-I at 10 ng/ml through Type IV Collagen

Antibody used	Cells migrating
none	357 +/- 26 (100%)
anti-α2	177 +/- 12 (49%)
anti-α3	475 +/- 55 (133%)
anti-β1	20 +/- 5 (6%)

Assay #136 all MDA-231 cells, migrating to IGF-I at 10 ng/ml through Laminin

Antibody used	Cells migrating
IgG (negative control)	842 +/- 50 (100%)
anti-α2	701 +/- 76 (83%)
anti-α3	698 +/- 82 (83%)

Assay #138 all MDA-231 cells, migrating to IGF-I at 10 ng/ml through Laminin

Antibody used	Cells migrating
IgG (negative control)	512 +/- 26 (100%)
anti-α2	414 +/- 38 (81%)
anti-α3	405 +/- 47 (79%)

Table 4. Results from assays using anti-integrin antibodies. The migration to IGF-I at 10 ng/ml is arbitrarily assigned a value of 100% for each assay. All data represents actual number of cells counted +/- SE followed by (%), the percentage migration compared to that observed in response to IGF-I at 10 ng/ml.

FIGURE 1

IGF-I Crosslinking Studies Using MCF-7 Cells
A Comparison of Cells Grown in Fetal Calf Serum Versus Calf Serum

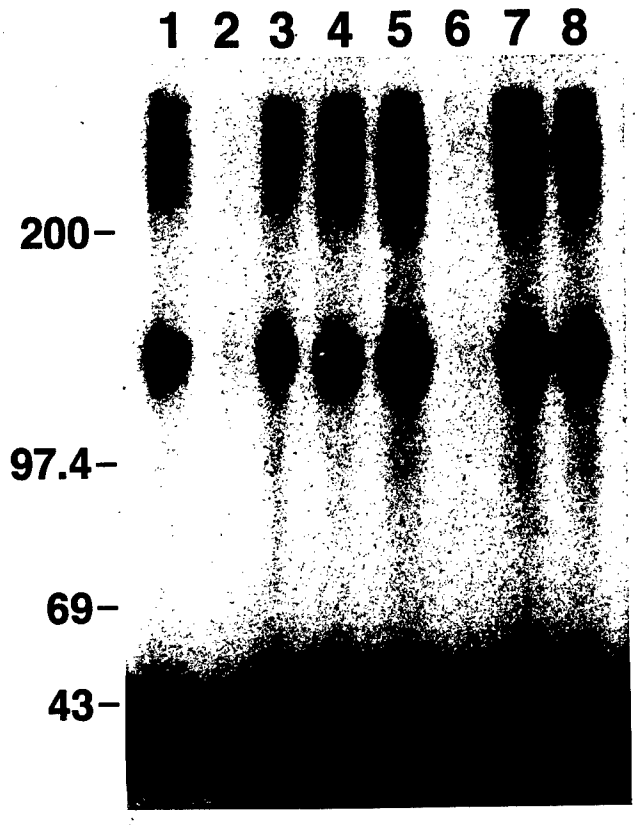

Figure 1. Crosslinking Studies for the Type I IGF receptor in MCF-7 cells. Lanes 1-4 contain MCF-7 cells grown always in 10% fetal calf serum. Lanes 5-8 contain cells exposed to 10% calf serum for 10 days. The band corresponding to approximately 133 kDa represents the Type I IGF receptor crosslinked to radiolabelled IGF-I. The cells loaded in lanes 2 and 6 were exposed to cold IGF-I (100 ng.ml) for competition of Type I receptor binding. Lanes 3 and 7 are from cells exposed to a high concentration of insulin (5 μg/ml) as a control.

TABLE 6
Migration Response of MCF-7 Cells to IGF-I and IGFBP-1

Assay #155		IGFBP-1 absent	present
	IGF-I		
	absent	no cells	no cells
	present	322 (100%)	384 (119%)
Assay #156		IGFBP-1 absent	present
	IGF-I		
	absent	no cells	no cells
	present	275 (100%)	292 (106%)
Assay #158		IGFBP-1 absent	present
	IGF-I		
	absent	no cells	no cells
	present	333 (100%)	365 (110%)
Assay #161		IGFBP-1 absent	present
	IGF-I		
	absent	no cells	no cells
	present	225 (100%)	243 (108%)
Assay #163		IGFBP-1 absent	present
	IGF-I		
	absent	no cells	no cells
	present	333 (100%)	365 (110%)

Table 5. Migratory response of MCF-7 cells to IGF-I with and without 60 minutes of pre-incubation with IGFBP-1. Migration to IGF-I alone (10 ng/ml) is arbitrarily assigned as 100% migration. For assays #155, 156 and 158, a 10 nM concentration of IGFBP-1 was used. For assays #161 and 163, the IGFBP-1 concentration was 50 nM.

TABLE 7

Migration Response of MDA-231 Cells to IGF-I and IGFBP-1

Assay #157		IGFBP-1 absent	present
	IGF-I		
	absent	231 (80%)	367 (127%)
	present	289 (100%)	395 (137%)

Assay #158		IGFBP-1 absent	present
	IGF-I		
	absent	343 (98%)	439 (126%)
	present	349 (100%)	560 (160%)

Assay #161		IGFBP-1 absent	present
	IGF-I		
	absent	494 (66%)	517 (69%)
	present	746 (100%)	539 (72%)

Table 7. Migratory response of MDA-231 cells to IGF-I with and without 60 minutes of pre-incubation with IGFBP-1. Migration to IGF-I alone (10 ng/ml) is arbitrarily assigned as 100% migration for each assay. For assays #157 and 158, a 10 nM concentration of IGFBP-1 was used. For assay #161, the IGFBP-1 concentration was 50 nM.

TABLE 8
Comparison of Effects of Native IGFBP-1 with its WGD Mutant

Assay #162 all MDA-231 cells

IGFBP-1 / IGF-I	absent	present	WGD mutant
absent	616 (89%)	577 (83%)	569 (82%)
present	695 (100%)	604 (87%)	695 (100%)

Assay #163 all MCF-7 cells

IGFBP-1 / IGF-I	absent	present	WGD mutant
absent	no cells	no cells	no cells
present	152 (100%)	278 (183%)	198 (130%)

Table 8. Results from initial studies comparing the effect of the WGD mutant form of IGFBP-1 to the native binding protein in the absence and presence of IGF-I. IGF-I was used in a 10 ng/ml concentration while the IGFBP-1 was 50 nM. Migration of the cells not pre-incubated with a binding protein migrating in response to IGF-I is arbitrarily assigned a value of 100% migration for each assay.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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DAMD17-96-1-6066	ADB235510
DAMD17-96-1-6029	ADB259877
DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
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DAMD17-99-1-9048	ADB258562
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DAMD17-98-C-8029	ADB261408
DAMD17-97-1-7299	ADB258750
DAMD17-97-1-7060	ADB257715
DAMD17-97-1-7009	ADB252283
DAMD17-96-1-6152	ADB228766
DAMD17-96-1-6146	ADB253635
DAMD17-96-1-6098	ADB239338
DAMD17-94-J-4370	ADB235501
DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754